Expression of the aging gene p66Shc is increased in peripheral blood monocytes of patients with acute coronary syndrome but not with stable coronary artery disease

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Abstract: OBJECTIVE: The interplay between oxidative stress and inflammation is crucial in the pathogenesis of atherosclerosis. The adaptor protein p66Shc is implicated in atherogenesis and oxidative stress related responses in animal models of diseases. However, its role in humans remains to be defined. In this study, we hypothesized that expression of p66Shc increases in peripheral blood monocytes of patients affected by acute coronary syndromes (ACS). METHODS: Male subjects aged 59±4 (mean±SD) years admitted for cardiac catheterization were subdivided in three groups: (a) no local stenosis for the control group, (b) at least one stenosis 75% in either left, circumflex or right coronary artery for the coronary artery disease (CAD) group or (c) ST-elevation/non-ST-elevation myocardial infarction for the ACS group. Monocytes were isolated from whole blood and p66Shc RNA levels were determined by quantitative real time PCR. RESULTS: p66Shc RNA levels were increased in ACS patients as compared to CAD (p=0.007) and controls (p=0.0249). Furthermore, malondialdehyde (MDA) and C-reactive protein (CRP) were increased in plasma of ACS patients. Levels of MDA correlated positively to p66Shc (r=0.376, p=0.01). Our data demonstrate increased p66Shc levels in monocytes of ACS but not CAD patients. CONCLUSION: This study suggests an involvement of p66Shc in the transition of a stable CAD to an ACS patient. p66Shc was associated with states of increased oxidative stress. Further work is needed to understand whether p66Shc may represent a possible pharmacological target or whether it represents an interesting novel biomarker.

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Abstract

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Methods: Male subjects aged 59 ± 4 (mean ± SD) years admitted for cardiac catheterization were subdivided in three groups: a) no local stenosis for the control group, b) at least one stenosis ≥ 75% in either left, circumflex or right coronary artery for the coronary artery disease (CAD) group or c) ST-elevation/non-ST-elevation myocardial infarction for the ACS group. Monocytes were isolated from whole blood and p66Shc RNA levels were determined by quantitative real time PCR.

Results: p66Shc RNA levels were increased in ACS patients as compared to CAD ($p = 0.007$) and controls ($p = 0.0249$). Furthermore, malondialdehyde (MDA) and C-reactive protein (CRP) were increased in plasma of ACS patients. Levels of MDA correlated positively to p66Shc ($r = 0.376$, $p = 0.01$). Our data demonstrate increased p66Shc levels in monocytes of ACS but not CAD patients.

Conclusion: This study suggests an involvement of p66Shc in the transition of a stable CAD to an ACS patient. p66Shc was associated with states of increased oxidative stress. Further work is needed to understand whether p66Shc may represent a possible pharmacological target or whether it represents an interesting novel biomarker.

Keywords: p66shc, oxidative stress, coronary artery disease, acute coronary syndrome

Expression of the Aging Gene p66Shc is Increased in Peripheral Blood Monocytes of Patients with Acute Coronary Syndrome but not with stable Coronary Artery Disease

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Running head: Elevation of p66Shc in ACS

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Introduction

Oxidative stress plays an important role in the pathogenesis of atherosclerosis, leading to cardiovascular complications such as myocardial infarction and stroke [1]. Classical cardiovascular risk factors (e.g. diabetes mellitus, dyslipidemia, smoking and age) increase systemic oxidative stress. As a consequence, nitric oxide availability decreases and endothelial dysfunction occurs as an early landmark of atherogenesis [2]. Oxidative stress triggers apoptosis, as seen in atherosclerotic plaques, and influences expression patterns of vascular cytokines and growth hormones, eventually resulting in a chronic inflammatory state typical of atherosclerosis [3]. Reactive oxygen species (ROS) are crucially involved in mediating the effects of inflammation by directly oxidizing target proteins and also by acting as second messengers in several redox responses [4]. Many insights into the underlying pathways have already been gained, yet the exact molecular mechanisms linking ROS formation to atherogenesis are poorly understood.

The adaptor protein p66Shc was first described as an aging gene in p66Shc$^{-/-}$ mice where its deletion was shown to increase life span by 30%. In later studies, p66Shc$^{-/-}$ mice developed less atherosclerotic plaques, reduced hyperglycemia-dependent endothelial dysfunction as well as decreased damage in a model of hind limb ischemia [3, 5-8].

p46Shc, p52Shc and p66Shc make up the family of ShcA adaptor proteins with molecular masses of 46, 52 and 66 kDa respectively. The 3 isoforms display a common Src-homology 2 domain, a collagen-homology region, and a phosphotyrosine-binding domain (SH2-CH1-PTB). However, the splice variant p66Shc contains a unique N-terminal region (CH2) which is crucial for its role as a redox enzyme implicated in mitochondrial ROS

formation [9]. The splice variant p66Shc has been shown to increase cellular ROS and mediate apoptosis [10]. After phosphorylation on Ser36 by oxidative stress induced protein kinase C beta (PKCβ), p66Shc translocates to the mitochondria. There, p66Shc affects electron transfer from cytochrome c to oxygen, leading to the formation of the radical H_20_2 [11]. Triggered by ROS, p66Shc also lowers FOX03a activity which results in a decreased expression of the scavengers catalase and manganese superoxide dismutase (MnSOD) [12]. Mitochondrial apoptosis involves the release of a variety of oxidant complexes from the mitochondrial intermembrane space into the cytoplasm. This process is mediated in part by permeability transition pore (PTP), a mitochondrial membrane channel protein. It is hypothesized that one of the downstream effects of p66Shc is the opening of this pore [11, 13]. Thus, p66Shc seems to be embedded in a system sensing cellular stresses and translating them into an increase of cellular ROS, eventually inducing the apoptotic cascade.

Overall, p66Shc is a promising therapeutic target for, and marker of ROS-related cardiovascular diseases. However, human data confirming this hypothesis are still scarce. It has been previously reported that p66Shc mRNA levels are higher in peripheral blood mononuclear cells of diabetics compared to healthy individuals [14]. Additionally, a recent study indicated an association between p66Shc mRNA expression and coronary atherosclerosis in humans [15].

In this study, we characterized p66Shc expression levels in peripheral blood monocytes of subjects with stable coronary artery disease (CAD) and acute coronary syndrome (ACS) compared to healthy individuals without angiographic signs of coronary atherosclerosis. Additionally, we evaluated whether p66Shc expression correlates to plasma levels of malondialdehyde (MDA), a marker of lipid peroxidation and systemic oxidative stress.

Materials and Methods

Patient data

Male subjects aged 54 to 65 years admitted to the cardiac catheterization laboratory at the University Hospital Zurich between December 2009 and September 2011 for coronary angiography were included in the study. Patients diagnosed with diabetes mellitus, as well as individuals suffering from an active neoplastic, infectious of autoimmune disease were not included in the study population. For the CAD group, a history of ACS < 6 months prior to the study was considered as an exclusion criterion. Ethical approval was granted by the institutional ethical committee. All subjects signed an informed consent form.

Data acquisition procedures

Blood withdrawal took place either during cardiac catheterization from the iliac artery or during routine sampling at hospital admission from the antecubital vein. The degree of coronary atherosclerosis was determined by quantitative coronary angiography by the interventional cardiologist performing the catheterization blinded to the results of the p66Shc expression level. Criteria for study group assignment were a) no angiographically identifiable local stenosis (>20%) or diffuse wall sclerosis for the control group, b) at least one stenosis ≥ 75% in either left, circumflex or right coronary artery for the CAD group and c) either a ST-elevation (typical chest pain and two ST segment elevations ≥ 0.1mV) or a non-ST-elevation (typical chest pain with ≥ four-fold elevation of Troponin T) myocardial infarction, both being admitted for percutaneous coronary intervention, for the ACS group.

All patients underwent a general clinical examination and the medical history was recorded. Previous medication was not discontinued for the purpose of this study. Blood pressure was measured in horizontal position using automatic blood pressure meters. Weight

was measured on hospital admission and BMI calculated accordingly. Smoking was defined as cigarette consumption of > 10 cigarettes / day. Blood parameters were measured on automated routine analyzers. High density lipoprotein cholesterol was measured on Cobas Integra (Roche). Plasma concentrations of AST, ALT, total cholesterol, triglycerides, C-reactive protein (CRP), creatinine, and glucose were measured on Modular Analytics (Roche) and Cobas 8000 (Roche).

Isolation of peripheral blood monocytes

Blood was collected in Ficoll tubes (Vacutainer CPT, BD Diagnostics) and centrifuged for 20 minutes at 1'800×g and room temperature. The turbid white layer above the Ficoll containing the mononuclear blood cells was transferred to a clean tube and washed twice with PBS. Subsequently, monocytes were isolated using magnetic CD14-coated beads and magnetic activated cell sorting (MACS). Finally, isolated cells were resuspended in TRIzol reagent (Invitrogen) and the total RNA extract was stored at -80°C.

Preparation of cDNA and quantitative real-time PCR

RNA was further purified from the TRIzol extract with 20% chloroform, centrifuged and precipitated from the resulting water phase with isopropanol. Subsequently, the RNA was washed with 70% ethanol, the RNA pellet was resuspended in diethylpyrocarbonate (DEPC) water and the RNA concentration was measured with a spectrophotometer (NanoDrop, Thermo Scientific). Reverse transcription of RNA was performed with the Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). RNA was heated at 65° Celsius for 10 minutes. 2 µg of RNA was reverse transcribed with random hexamer primers (pd(N)6; 1 µg/µl) (TaKaRa) at 37° Celsius for 1 hour. Finally, quantitative real-time PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems™) on a 7500 Fast Real-Time

PCR System (Applied Biosystems™). Each reaction contained cDNA, p66shc specific TaqMan® MGB probes and primers (specific for the CH2 region) (20x) with a FAM™ reporter dye (predesigned by Applied Biosystems™), TaqMan® Universal PCR Master Mix (2x) (containing AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, passive reference, and optimized buffer components) and H_2O. Samples were run in duplicate and the human p66shc gene expression data were normalized against human 18S rRNA levels. Quantification was performed using $2^{-\Delta\Delta Ct}$ method.

Measurement of malondialdehyde

MDA, an end product of lipid peroxidation, is an established marker for oxidative stress. Plasma levels of MDA were measured using the thiobarbituric acid-reactive substances (TBARS) assay, as described previously [16].

Statistical analysis

Continuous variables are expressed as median (interquartile range) and frequencies for categorical variables. p66Shc mRNA levels were analyzed by the nonparametric Kruskal-Wallis-H test, followed by the Mann-Whitney-U test. Clinical data was analyzed by the χ^2 test for categorical and by the Mann-Whitney-U test for continuous data. Spearmans's correlation analysis was used to assess correlation between variables. In figures, data are displayed as boxplot (whiskers after tukey and median). The inclusion of 14 patients in each group, for a α-value of 0.05 (two-tailed; assuming a difference in densitometric unit of 0.1 and a standard deviation of 0.08), has the power to detect a significant difference of 91%. The level of significance was defined as a two-tailed p value < 0.05. All statistical analyses were performed with SPSS Statistics 19.0 for Windows (SPSS, Inc. 2010).

Results

Patients characteristics

In this study, expression of p66Shc mRNA was measured in monocytes isolated from blood from 18 ACS patients, 19 CAD patients and 16 age-matched, angiographically confirmed healthy controls. Overall median age was 61 years and all patients were of caucasian ethnicity. In the CAD group, 4 subjects (21%) had one, 4 (21%) had two and 11 (58%) had three vessel disease. The ACS group consisted of 7 (39%) STEMI and 11 (61%) NSTEMI patients, of which 9 (50%) had an occlusion of the left anterior, 3 (16%) of the circumflex and 6 (33%) of the right coronary artery. Myocardial infarction has been documented with electrocardiography, angiographic findings and laboratory markers of myocardial necrosis, i.e. troponin t and creatine kinase. Clinical data profile differed between groups in smoking status, aspirin, clopidogrel and statin usage, blood glucose, total cholesterol, and LDL levels (Table 1).

p66Shc expression is increased in patients with acute coronary syndrome

p66Shc mRNA levels were significantly higher in ACS patients (2.84×10^{-5} [2.1×10^{-5}–5.53×10^{-5}], n = 18) compared to healthy controls (2×10^{-5} [1.19×10^{-5}-2.78×10^{-5}], n = 16; p = 0.0249) or compared to CAD patients (1.73×10^{-5} [0.98×10^{-5}–2.23×10^{-5}], n = 19; p = 0.007) (Figure 1). However, expression levels of p66Shc did not differ significantly between CAD patients and healthy controls (p = 0.367) (Figure 1).

p66Shc expression correlates to levels of lipid peroxidation

MDA plasma levels measured by TBARS assay were significantly increased in ACS patients (1.261 [1.193-1.297], n = 15) compared to healthy controls (1.186 [1.164-1.236], n = 14; p = 0.029) and to CAD patients (1.135 [1.120-1.161], n = 17; p = 0.0002) (Figure 2). MDA levels in CAD patients were lower than in healthy controls (p = 0.006) (Figure 2). The MDA plasma concentration correlated significantly with p66Shc mRNA expression levels (r = 0.376; p = 0.01) (Figure 3).

CRP levels were significantly elevated in ACS patients (3.3 [1.4–5.3], n = 18) in comparison with healthy controls (1.1 [0.6–1.9], n= 16; p = 0.001) as well as in comparison with CAD patients (1.6 [0.7-2.5], n = 17; p = 0.006) (Figure 4). However, no significant difference in CRP plasma concentrations was found between CAD patients and healthy controls (p = 0.481).

Discussion

In this study, we report elevated levels of p66Shc mRNA in peripheral blood monocytes of patients who suffered from an acute myocardial infarction but not in those who display stable CAD. Plasma levels of MDA, an established marker of lipid peroxidation and thus oxidative stress, correlated positively to p66Shc expression. These findings allow the speculation that p66Shc may be an interesting novel biomarker associated to adverse cardiovascular events or that p66Shc may even play a role in mediating acute vascular complications rather than in the preceding chronic phase of coronary atherosclerosis. Surely enough, further work made on a prospective basis will be needed to address the above.

In 2005, Pagnin *et al.* [14] reported an elevation of p66Shc mRNA in peripheral blood monocytes of patients with diabetes type 2 compared to non-diabetic controls indicating that p66Shc is responsive to conditions of elevated ROS in humans. We therefore excluded diabetic patients to avoid diabetes as a possible confounding factor for p66Shc expression levels. Furthermore, the authors described that p66Shc mRNA levels correlated to plasmatic levels of 8-isoprostane, a marker of systemic oxidative stress. A recent study by Noda *et al.* [15] explored the association of p66Shc mRNA expression with CAD. The authors compared angiographically confirmed controls without CAD to CAD patients but did not include an ACS group. In that study, a higher expression of monocytic p66Shc mRNA was measured in the CAD group as compared to the healthy control group. However, the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) was not found to be increased in CAD patients and did not correlate with p66Shc mRNA levels. These data appear in contradiction to our data and those reported by Pagnin *et al.* where levels of p66Shc were only reported to be increased in conditions of increased oxidative stress. Furthermore, in line with the above, we report unchanged p66Shc levels in CAD patients.

In the study by Noda *et al.*, diabetes was not considered as an exclusion criterion and the proportion of patients on diabetes treatment was doubled in the CAD group compared to controls. Based on the previous study by Pagnin *et al*., this might explain why Noda *et al*. reported an elevated p66Shc expression in the CAD group. In the here reported study, diabetic patients were excluded, as confirmed by the comparable HbA1c levels observed between the three groups. The increased blood glucose levels found in the ACS group in our study are likely due to transient hyperglycemia during acute myocardial infarction, as frequently reported before [17]. Other differences in the study populations such as ethnicity, mean age, inclusion of females and use of medication could account in part for the discrepant results.

The elevated plasma MDA levels that we observed in the ACS group are in agreement with previous studies showing increased levels of systemic oxidative stress measured by different markers in the context of myocardial infarction, before as well as after revascularization [18-20]. p66Shc has been shown to be involved in ROS regulation in vitro [11, 13] and in vivo [7]. Thus, the question arises whether p66Shc plays a role in determining the fate of a CAD patient with respect to the development of acute complications such as myocardial infarction. In the past, cell death in atherosclerotic plaques has been thought to be of necrotic nature [21]. However, it was recently shown that also apoptosis of different cell types is largely prevalent at plaque sites and may contribute to plaque cap destabilization and consequent plaque rupture [22]. In a histological study, apoptosis of macrophages and foam cells was increased at locations of plaque rupture [23]. Furthermore, apoptosis of vascular smooth muscle cells has been shown to destabilize atherosclerotic plaques [24]. Consequently, the balance between proliferation and death of key cells in the atherosclerotic plaque may determine a shift to a more athero- and thrombogenic state [25]. p66Shc is tightly linked to apoptosis; it plays an essential role in p53-dependent apoptosis in mice embryo fibroblasts and furthermore, its overexpression leads to a higher rate of apoptosis in the same

cells [6, 13]. In aortic sections of p66Shc knockout mice fed a high fat diet, the frequency of vascular cell apoptosis was lower compared to wildtype [7]. The here reported data demonstrate an acute rise in p66Shc and ROS levels in ACS patients but not in CAD ones. Thus, it could be hypothesized that p66shc, through its proapoptotic properties, is involved in the acute destabilization of a stable CAD patient.

Markers of oxidative stress such as MDA or 8-isoprostane have been previously correlated to the presence of CAD [26]. However, our study did not confirm this finding. This may be explained by good control of risk factors in the study population, as demonstrated by intensive medical therapy, levels of cholesterol and frequency of smoking (Table 1). Furthermore, different studies reported oxidative stress lowering effects of commonly used pharmaceuticals for CAD patients such as statins, aspirin and beta-blockers [27, 28]. In the CAD group included in this study, aspirin and statin usage was significantly increased compared to the other groups. Differences in the intake of beta-blocking agents were not significant but notably, relative frequency was still nearly doubled between the control and the CAD patients.

In this study, CRP levels were increased in the ACS group compared to the control group and the CAD group. Previous reports demonstrated that CRP is an independent risk factor for ACS and in line with this notion CRP has been included in algorithms for risk prediction. As described above, the finding that CRP was not elevated in the CAD group could be due to good control of risk factors. An association between levels of CRP and p66Shc mRNA was not observed in the present study. Thus, our data do not support a direct link between p66Shc expression and inflammation but rather only a specific correlation to increased ROS levels.

In addition, we found a significant correlation between plasma levels of LDL cholesterol and p66Shc mRNA expression in monocytes (data not shown). This finding is in

line with previously reported data showing a correlation between plasmatic levels of LDL cholesterol and p66Shc mRNA in white blood cells of pacemaker patients [29].

There is a multitude of contributing factors to the development of atherosclerosis and, complete elucidation of all molecular mechanisms is a very complex task. The few clinical data currently available on p66Shc's role in human cardiovascular disease underscore the need for further clinical research to address it. Atherosclerosis is currently regarded as an inflammatory disease with immune cells such as monocytes and macrophages as established key players [30]. For this reason and because of the simple acquisition procedure from blood samples, peripheral blood monocytes were selected for this study. Measuring p66Shc in endothelial cells or in atherosclerotic plaque tissue would offer interesting additional insights, yet sample acquisition from humans poses practical and ethical barriers.

In summary, our data suggest that p66Shc may be an interesting novel biomarker associated to major adverse cardiovascular events; furthermore, p66Shc may play a role in mediating the transition between a stable CAD and an acute ACS patient. Further prospective studies will be needed to address this hypothesis.

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References

[1] Madamanchi, NR, Vendrov, A and Runge, MS, Oxidative stress and vascular disease, Arterioscler Thromb Vasc Biol, 2005;25:29-38.

[2] Davignon, J and Ganz, P, Role of endothelial dysfunction in atherosclerosis, Circulation, 2004;109:III27-32.

[3] Francia, P, Cosentino, F, Schiavoni, M, et al., p66(Shc) protein, oxidative stress, and cardiovascular complications of diabetes: the missing link, J Mol Med, 2009;87:885-891.

[4] Rhee, SG, Cell signaling. H2O2, a necessary evil for cell signaling, Science, 2006;312:1882-1883.

[5] Camici, GG, Schiavoni, M, Francia, P, et al., Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress, Proc Natl Acad Sci U S A, 2007;104:5217-5222.

[6] Migliaccio, E, Giorgio, M, Mele, S, et al., The p66shc adaptor protein controls oxidative stress response and life span in mammals, Nature, 1999;402:309-313.

[7] Napoli, C, Martin-Padura, I, de Nigris, F, et al., Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet, Proc Natl Acad Sci U S A, 2003;100:2112-2116.

[8] Zaccagnini, G, Martelli, F, Magenta, A, et al., p66(ShcA) and oxidative stress modulate myogenic differentiation and skeletal muscle regeneration after hind limb ischemia, J Biol Chem, 2007;282:31453-31459.

[9] Camici, GG, Shi, Y, Cosentino, F, et al., Anti-Aging Medicine: Molecular Basis for Endothelial Cell-Targeted Strategies - A Mini-Review, Gerontology, 2010.

[10] Gertz, M and Steegborn, C, The Lifespan-regulator p66Shc in mitochondria: redox enzyme or redox sensor?, Antioxid Redox Signal, 2010;13:1417-1428.

[11] Giorgio, M, Migliaccio, E, Orsini, F, et al., Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis, Cell, 2005;122:221-233.

[12] Nemoto, S and Finkel, T, Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway, Science, 2002;295:2450-2452.

[13] Trinei, M, Giorgio, M, Cicalese, A, et al., A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis, Oncogene, 2002;21:3872-3878.

[14] Pagnin, E, Fadini, G, de Toni, R, et al., Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: relationship to oxidative stress, J Clin Endocrinol Metab, 2005;90:1130-1136.

[15] Noda, Y, Yamagishi, S, Matsui, T, et al., The p66shc gene expression in peripheral blood monocytes is increased in patients with coronary artery disease, Clin Cardiol, 2010;33:548-552.

[16] Girotti, MJ, Khan, N and McLellan, BA, Early measurement of systemic lipid peroxidation products in the plasma of major blunt trauma patients, J Trauma, 1991;31:32-35.

[17] Loomba, RS and Arora, R, Hyperglycemia and acute coronary syndrome: a systematic review of hyperglycemia's impact on ACS, Am J Ther, 2010;17:e48-51.

[18] Berg, K, Jynge, P, Bjerve, K, et al., Oxidative stress and inflammatory response during and following coronary interventions for acute myocardial infarction, Free Radic Res, 2005;39:629-636.

[19] Dousset, JC, Trouilh, M and Foglietti, MJ, Plasma malonaldehyde levels during myocardial infarction, Clin Chim Acta, 1983;129:319-322.

[20] Kaminski, K, Bonda, T, Wojtkowska, I, et al., Oxidative stress and antioxidative defense parameters early after reperfusion therapy for acute myocardial infarction, Acute Card Care, 2008;10:121-126.

[21] Woolf, N, Pathology of atherosclerosis, Br Med Bull, 1990;46:960-985.

[22] Bjorkerud, S and Bjorkerud, B, Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability, Am J Pathol, 1996;149:367-380.

[23] Kolodgie, FD, Narula, J, Burke, AP, et al., Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death, Am J Pathol, 2000;157:1259-1268.

[24] Halvorsen, B, Otterdal, K, Dahl, TB, et al., Atherosclerotic plaque stability--what determines the fate of a plaque?, Prog Cardiovasc Dis, 2008;51:183-194.

[25] Geng, YJ and Libby, P, Progression of atheroma: a struggle between death and procreation, Arterioscler Thromb Vasc Biol, 2002;22:1370-1380.

[26] Kotur-Stevuljevic, J, Memon, L, Stefanovic, A, et al., Correlation of oxidative stress parameters and inflammatory markers in coronary artery disease patients, Clin Biochem, 2007;40:181-187.

[27] Carnevale, R, Pignatelli, P, Di Santo, S, et al., Atorvastatin inhibits oxidative stress via adiponectin-mediated NADPH oxidase down-regulation in hypercholesterolemic patients, Atherosclerosis, 2010;213:225-234.

[28] Nascimento-Silva, V, Arruda, MA, Barja-Fidalgo, C, et al., Aspirin-triggered lipoxin A4 blocks reactive oxygen species generation in endothelial cells: a novel antioxidative mechanism, Thromb Haemost, 2007;97:88-98.

[29] Bosutti, A, Grassi, G, Zanetti, M, et al., Relation between the plasma levels of LDL-cholesterol and the expression of the early marker of inflammation long pentraxin PTX3 and

the stress response gene p66ShcA in pacemaker-implanted patients, Clin Exp Med, 2007;7:16-23.

[30] Libby, P, Okamoto, Y, Rocha, VZ, et al., Inflammation in atherosclerosis: transition from theory to practice, Circ J, 2010;74:213-220.

Figure captions

Figure 1. p66Shc mRNA expression, relative to expression of 18S rRNA, in peripheral blood monocytes from angiographically confirmed CAD-free controls, stable CAD patients and patients with ACS.

Figure 2. Plasma concentrations of the oxidative stress marker malondialdehyde (MDA) in the three patient groups.

Figure 3. Scatterplot of monocytic p66Shc mRNA expression, relative to ribosomal protein S18, and plasma levels of malondialdehyde (MDA) ($r = 0.376$, $p = 0.01$).

Figure 4. C-reactive protein (CRP) plasma concentrations in the three patient groups.

Table legends

Table 1. Characteristics of study participants.

Point by point response

Reviewer #1:

Q1. In the method section, the authors have not indicated which specific primers and probe they used for p66shc mRNA quantification. Did they use probes specific for CH2 region of SHC gene? This should be included

A1. For the determination of p66shc gene levels we used a primer/probe mix specific for p66shc, predesigned by Applied Biosystems™. The primer/probe anneals within the first 350 bp region of p66shc cDNA, indeed the CH2 region as pointed out by the reviewer. This 350 bp are unique and specific for p66shc. This information has now been added to the relevant M&M section in the manuscript.

Q2. In the method section, the authors wrote that they used ribosomal protein S18 mRNA levels to normalize the data in p66shc quantification which has not the same expression of p66shc since this latter is really low expressed in leukocytes. Usually, it is better to use housekeeping genes with an expression level similar to the one of the gene of interest and this is not the case since p66shc in leukocytes is really low expressed. Did the authors try also other housekeeping genes?

A2. First of all we would like to apologize to the reviewer since, following his valuable comment we found that we misspelled the type of housekeeping gene we used. Indeed, we used 18S rRNA levels (not ribosomal protein S18 mRNA as mentioned in the first version of the manuscript) to normalize our p66shc gene expressions.

One of the most important steps in gaining reliable data for the determination of gene levels using quantitative PCR, is the selection of an appropriate endogenous control (housekeeping gene). Generally, any gene shown to be expressed at the same level in all study samples can potentially be used as an endogenous control. Nevertheless, it is critical to determine if the study design is affecting the level of the endogenous gene.

In our study, we used 18S rRNA levels to normalize our p66shc gene expressions. 18S rRNA is a widely used endogenous control and it is known to be uniformly expressed in cells. In our study, only small variations of 18S rRNA levels between the samples were observed, also indicating a uniform expression of this gene in our experimental setup. Although, no other housekeeping genes were tested, we are confident to assume equal results if other housekeeping genes would be used for the normalisation.

Q3. MDA is a well known oxidative stress marker but it is also subjected to many interferences and other markers such as 8-isoprostanes are preferable.

A3. We additionally investigated concentrations of 8-isoprostane by analyzing frozen plasma samples of our patients, as requested. Unfortunately however, the commercially available competitive

immunoassay (Cayman Chemicals, USA) measured no relevant levels of 8-isoprostane in all our samples. Indeed, 8-isoprostane concentrations in all groups were up to 10^2-fold lower than reported in previous studies[1-3] (Figure 1, lower panel). We explain those results by the fact that no antioxidative agents such as butylated hydroxytoluene (BHT) or indomethacine (to prevent the ex vivo formation of other prostaglandins that might interfere with the assay), were added to our samples before storage, which is highly recommended by the producer for carrying out 8-isoprostane measurements posthoc. Since additional analysis of indicators of oxidative stress was not planned originally, no such agents

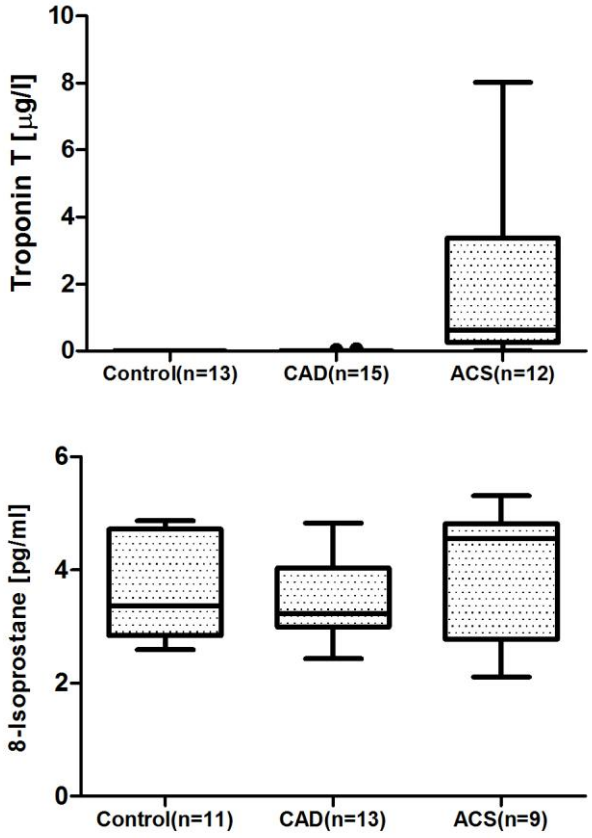

Figure 1. Plasma levels of troponin t and 8-isoprostane by patient group

preventing degradation of oxidation products were added during preparation of samples for storage.

However, to reinforce our argumentation for an increased ROS level in ACS patients, the following points can be considered.

The presence of raised levels of 8-isoprostane in ACS patients is an established notion[1,4]. Documentation of acute coronary events in the ACS patients enrolled in the present study is very thorough with electrocardiography, radiological findings on coronary angiography and quantitative results of laboratory markers for the diagnosis of myocardial infarction (troponin t). In line with this, troponin t levels were increased in the acs group (Figure 1, upper panel). The reference range for troponin t in the laboratory where our analyses have been performed is 0-0.014µg/l and anything above is considered pathological.

Therefore, we are very confident that those patients did indeed suffer an ACS which is inevitably associated with increased ROS. Even though it was not possible to provide reliable data on 8-

isoprostance from our samples, it can be assumed that those levels were increased at the moment of blood withdrawal from subjects in the ACS group.
Additionally, a positive correlation between p66Shc mRNA expression and total plasma 8-isoprostanes was previously reported by Pagnin E et al.[5] in a study comparing diabetic to non-diabetic patients. This finding by another group further supports our hypothesis that p66Shc mRNA and oxidative stress are associated and we provide evidence for this with a different marker of oxidative stress, namely MDA.
Additionally, there is evidence showing a good correlation of MDA levels to 8-isoprostanes in vivo[6] and MDA has been shown to discriminate well and with improved accuracy over 8-isoprostane between different states of oxidative stress during cardiac surgery[7].
The evaluation of an additional marker of oxidation in this study would have certainly given further support to our data but given the issues discussed above, we feel it is not indispensable for reaching our conclusion.

Q4. The authors found a lower MDA level in CAD patients compared to controls, which is not perfectly in agreement with literature, as they reported. They explained this discrepancy with a good control of the patients and the use of statins and aspirin but, in table 1, it is indicated also the use of <beta>-blockers for the 53% of the CAD patients versus the 27% and 21% in controls and ACS patients respectively. It is well demonstrated that many anti-hypertensive drugs have antioxidant effects and also <beta>-blockers show this peculiarity. This could be in part an explanation for the "abnormal" MDA values in these patients and should be indicated in the discussion session.
We thank the reviewer for this valuable comment. The low MDA concentrations in stable CAD patients cannot be explained with absolute certainty, as conclusions about causality cannot be drawn with an observational study design like the present one. In general, questions about causal relations in oxidative stress pathways are extremely challenging in humans. As reviewer #1 pointed out, oxidative stress lowering effects of different pharmaceuticals prescribed for CAD patients are well established; the explanatory hypothesis of decreased MDA levels due to a significantly increased intake of multiple oxidative stress lowering medications in the CAD group seems reasonable.
The difference in frequency of beta-blockers intake between the groups studied was below the level of significance; e.g. the chi-squared test comparing the numbers of beta-blocker users between stable CAD patients and ACS patients resulted in a p value of 0.10, even though the relative frequency of beta-blocker intake was more than doubled in the CAD group as compared to ACS. Following the suggestion of the reviewer, we have now included beta-blockers in the appropriate paragraph of the discussion where we describe the possible influence of pharmaceutical on levels of oxidative stress in the CAD group (changes in the manuscript are shown in red).

Reviewer #2:

The authors should be commended for this manuscript and this reviewer has only positive comments about this manuscript.

Reviewer #3:

Q1. Sample size is small and should be increased to confirm data and correlation.

A1. A sample size calculation based the decision on the number of patients to be included and the final results showed statistical significance. Nonetheless, we agree with the comment of the reviewer and we recruited additional patients throughout the time we had available for submitting the revisions of the present study.

The sample size in the 3 experimental groups included in this study was raised from 15, 17 and 14 in healthy, CAD and ACS respectively to 16, 19 and 18.

The authors would like to add that compared to similar studies where the levels of p66Shc were assessed in diabetic (Pagnin, et al.[5]) or CAD patients (Noda, et al.[8]), the number of patients/group included in this study now is higher or equal, respectively.

Q2. As MDA provided somehow inconsistent results (lower levels in CAD vs controls remain essentially unexplained), at least another plasma marker of oxidative stress should be determined in parallel (i.e. TBARS, isoprostanes, SOD, catalase, ...). This should be easily done using frozen samples..

A2. We thank the reviewer for this valuable comment. The low MDA concentrations in stable CAD patients cannot be explained with absolute certainty, as conclusions about causality cannot be drawn with an observational study design like the present one. In general, questions about causal relations in oxidative stress pathways are extremely challenging in humans.

Oxidative stress lowering effects of different drugs prescribed for CAD patients are well established; thus, the argument of decreased MDA levels due to a significantly increased intake of multiple oxidative stress lowering medications (e.g statins, beta blockers and aspirin) in the CAD group seems reasonable.

Nevertheless, as requested by the reviewer we performed 8-isoprostane measurements in the 3 experimental groups included in this study so as to provide further evidence for an augmented ROS production in the ACS group.

Unfortunately however, the commercially available competitive immunoassay (Cayman Chemicals, USA) measured no relevant levels of 8-isoprostane in all our samples (Figure 1, lower panel). Indeed, 8-isoprostane concentrations in all groups were up to 10^2-fold lower than reported in previous studies[1-3]. We explain those results by the fact that no antioxidative agents such as butylated hydroxytoluene (BHT) or indomethacine (to prevent the ex vivo formation of other prostaglandins that might interfere with the assay), were added to our samples before storage, which is highly recommended by the producer for carrying out 8-isoprostane measurements posthoc. Since additional analysis of indicators of oxidative stress was not planned originally, no such agents preventing degradation of oxidation products were added during preparation of samples for storage.

However, to reinforce our argumentation for an increased ROS level in ACS patients, the following points can be considered.

The presence of raised levels of 8-isoprostane in ACS patients is an established notion[1,4]. Documentation of acute coronary events in the ACS patients enrolled in the present study is very thorough with electrocardiography, radiological findings on coronary angiography and quantitative results of laboratory markers for the diagnosis of myocardial infarction (troponin t). In line with this, troponin t levels were increased in the acs group (Figure 1, upper panel). The reference range for troponin t in the laboratory where our analyses have been performed is 0-0.014µg/l and anything above is considered pathological.

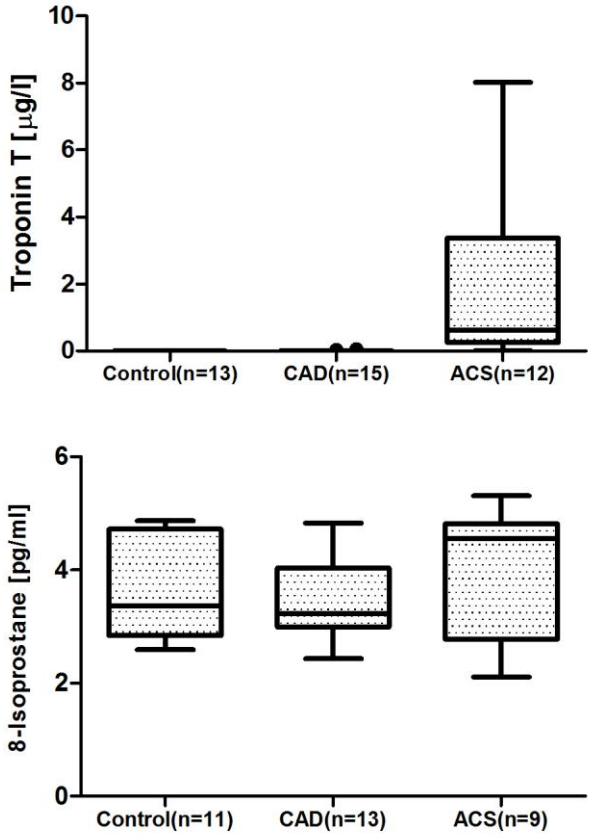

Figure 1. Plasma levels of troponin t and 8-isoprostane by patient group

Therefore, we are very confident that those patients did indeed suffer an ACS which is inevitably associated with increased ROS. Even though it was not possible to provide reliable data on 8-isoprostance from our samples, it can be assumed that those levels were increased at the moment of blood withdrawal from subjects in the ACS group.

Additionally, a positive correlation between p66Shc mRNA expression and total plasma 8-isoprostanes was previously reported by Pagnin E et al.[5] in a study comparing diabetic to non-diabetic patients. This finding by another group further supports our hypothesis that p66Shc mRNA and oxidative stress are associated and we provide evidence for this with a different marker of oxidative stress, namely MDA.

Additionally, there is evidence showing a good correlation of MDA levels to 8-isoprostanes in vivo[6] and MDA has been shown to discriminate well and with improved accuracy over 8-isoprostane between different states of oxidative stress during cardiac surgery[7].

The evaluation of an additional marker of oxidation in this study would have certainly given further support to our data but given the issues discussed above, we feel it is not indispensable for reaching our conclusion

Q3. Was there any correlation between p66Shc gene expression and CRP? If not, it may be worth stressing that oxidative stress and not inflammation is related to p66Shc biology.

A3. We fully agree with the statement of the reviewer in fact, no correlation between p66 and CRP was present in our study. In line with this, we have rephrased the relevant parts of the discussion where this aspect is argued (changes in the discussion are shown in red).

References mentioned in this response:

1. Wang B, Pan J, Wang L, Zhu H, Yu R, Zou Y. Associations of plasma 8-isoprostane levels with the presence and extent of coronary stenosis in patients with coronary artery disease. Atherosclerosis 2006;184:425-30.
2. Mueller T, Dieplinger B, Gegenhuber A, et al. Serum total 8-iso-prostaglandin F2alpha: a new and independent predictor of peripheral arterial disease. J Vasc Surg 2004;40:768-73.
3. Vassalle C, Botto N, Andreassi MG, Berti S, Biagini A. Evidence for enhanced 8-isoprostane plasma levels, as index of oxidative stress in vivo, in patients with coronary artery disease. Coron Artery Dis 2003;14:213-8.
4. LeLeiko RM, Vaccari CS, Sola S, et al. Usefulness of elevations in serum choline and free F2)-isoprostane to predict 30-day cardiovascular outcomes in patients with acute coronary syndrome. Am J Cardiol 2009;104:638-43.
5. Pagnin E, Fadini G, de Toni R, Tiengo A, Calo L, Avogaro A. Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: relationship to oxidative stress. J Clin Endocrinol Metab 2005;90:1130-6.
6. Ahmadzadehfar H, Oguogho A, Efthimiou Y, Kritz H, Sinzinger H. Passive cigarette smoking increases isoprostane formation. Life Sci 2006;78:894-7.
7. Veglia F, Werba JP, Tremoli E, et al. Assessment of oxidative stress in coronary artery bypass surgery: comparison between the global index OXY-SCORE and individual biomarkers. Biomarkers 2009;14:465-72.
8. Noda Y, Yamagishi S, Matsui T, et al. The p66shc gene expression in peripheral blood monocytes is increased in patients with coronary artery disease. Clin Cardiol 2010;33:548-52.

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Zurich, October 4th 2011

RE: Rebuttal letter manuscript no: Ref.: Ms. No. ATH-D-11-00732

Dear Dr. Fadini

Thank you for taking our manuscript into consideration. We reassessed all critical points raised by the reviewers and are pleased to resubmit our paper as well as the answers to the respective questions.

Although we addressed the vast majority of the points raised by the reviewers, we were faced with some technical problems concerning the requested 8-isoprostane measurement. Indeed, our samples had not been stored with antioxidants rendering them unsuitable for new isoprostane measurements (which we anyway tried to perform as shown in the point by point response). Nevertheless, we offer additional evidence to support elevated ROS levels in the ACS patients we enrolled in this study and we are convinced that such information will address the reviewer's concerns on this issue.

Changes in the manuscript are highlighted in red as required in the instructions to authors and responses to specific questions are given below.

Very best regards,

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Giovanni G. Camici

*Statement of Originality

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Zurich, October 6th 2011

Statement of Orginiality

Hereby, the authors affirm that this article is not under consideration for publication elsewhere. The publication of the manuscript has been approved by all authors listed and by the responsible authorities at the respective institutions. In case the article is accepted, it will not be published elsewhere by the authors.
The authors declare no conflict of interest to any of the data presented in this manuscript.

Yours sincerely

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Giovanni G. Camici

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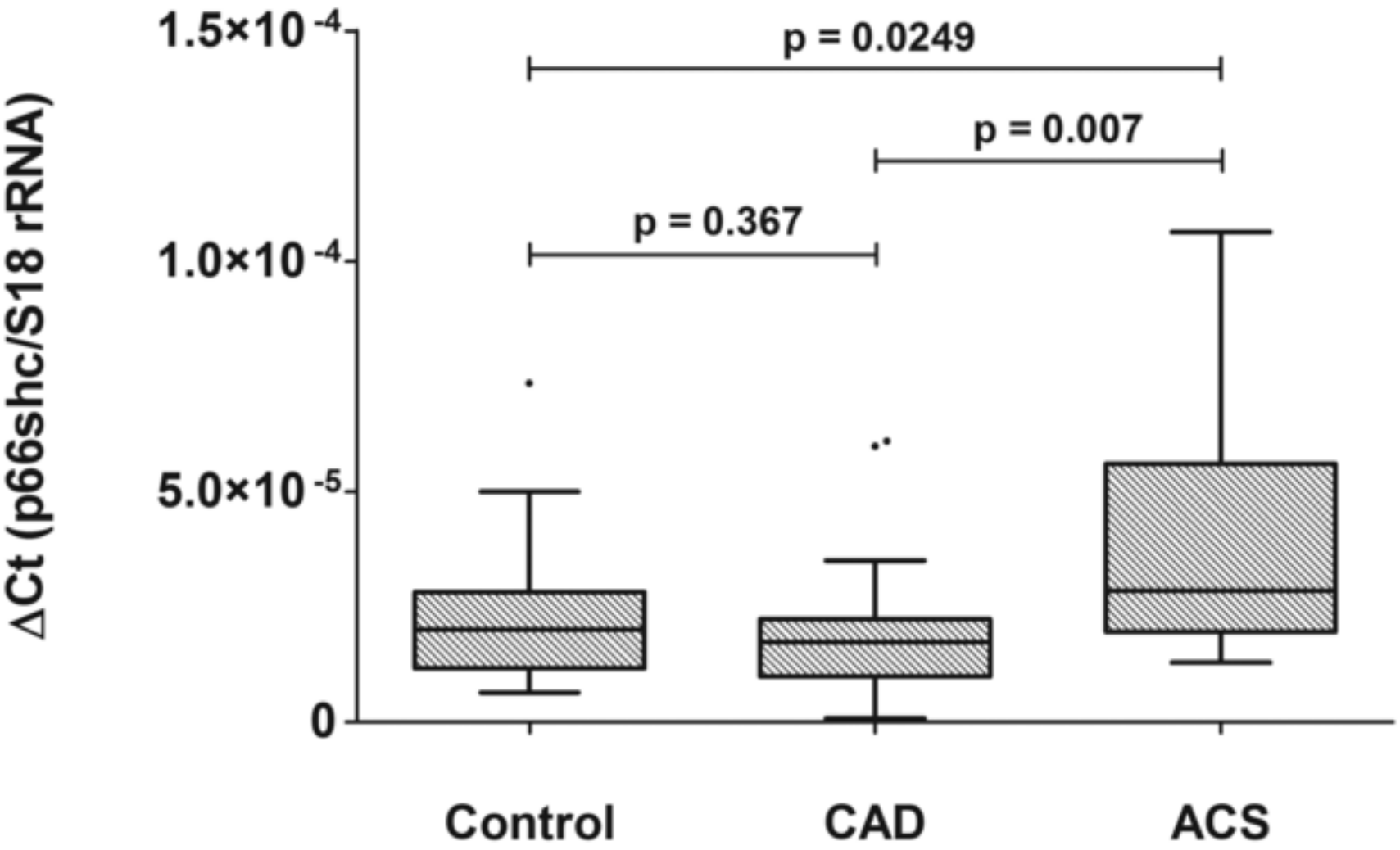

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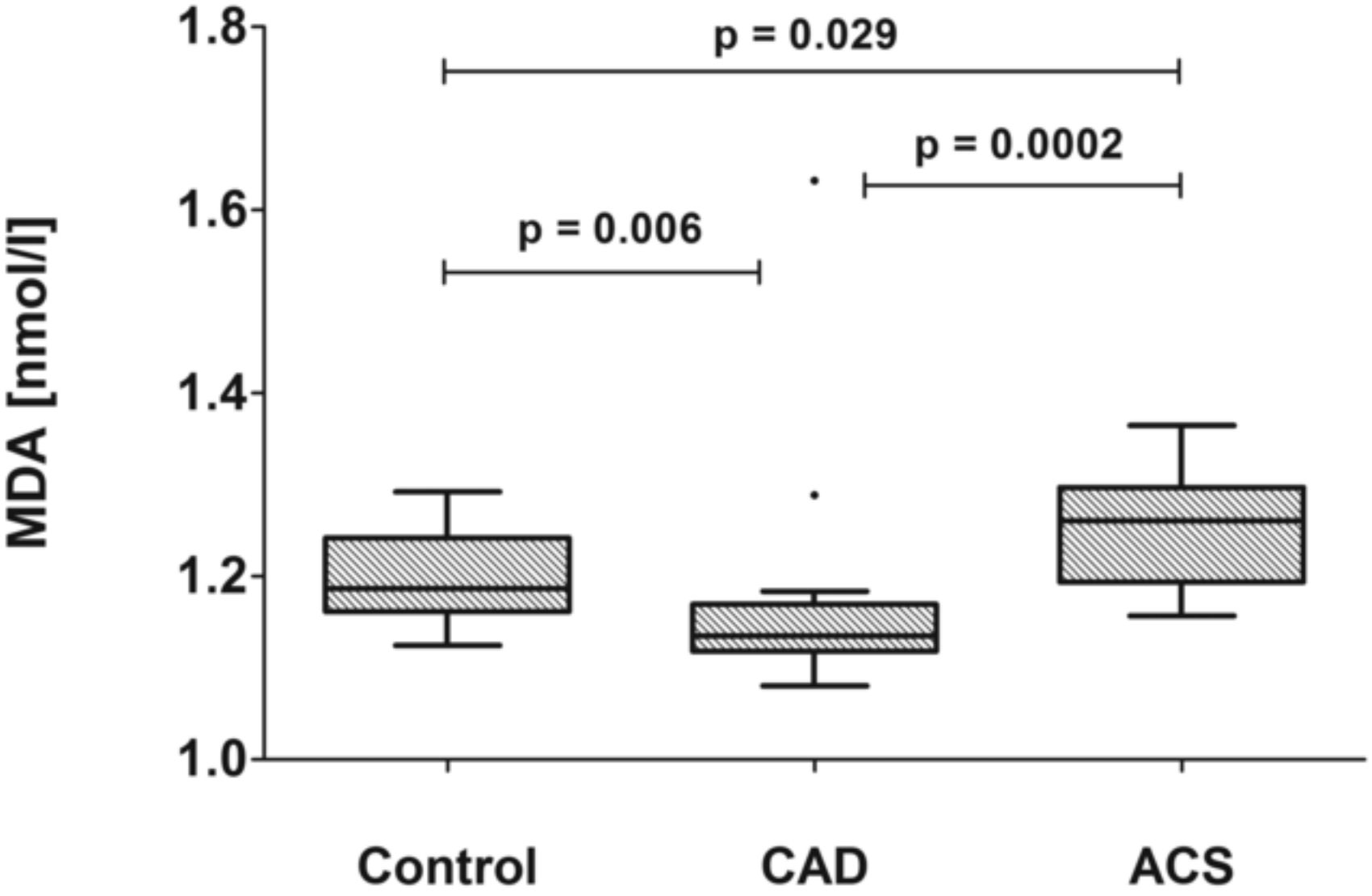

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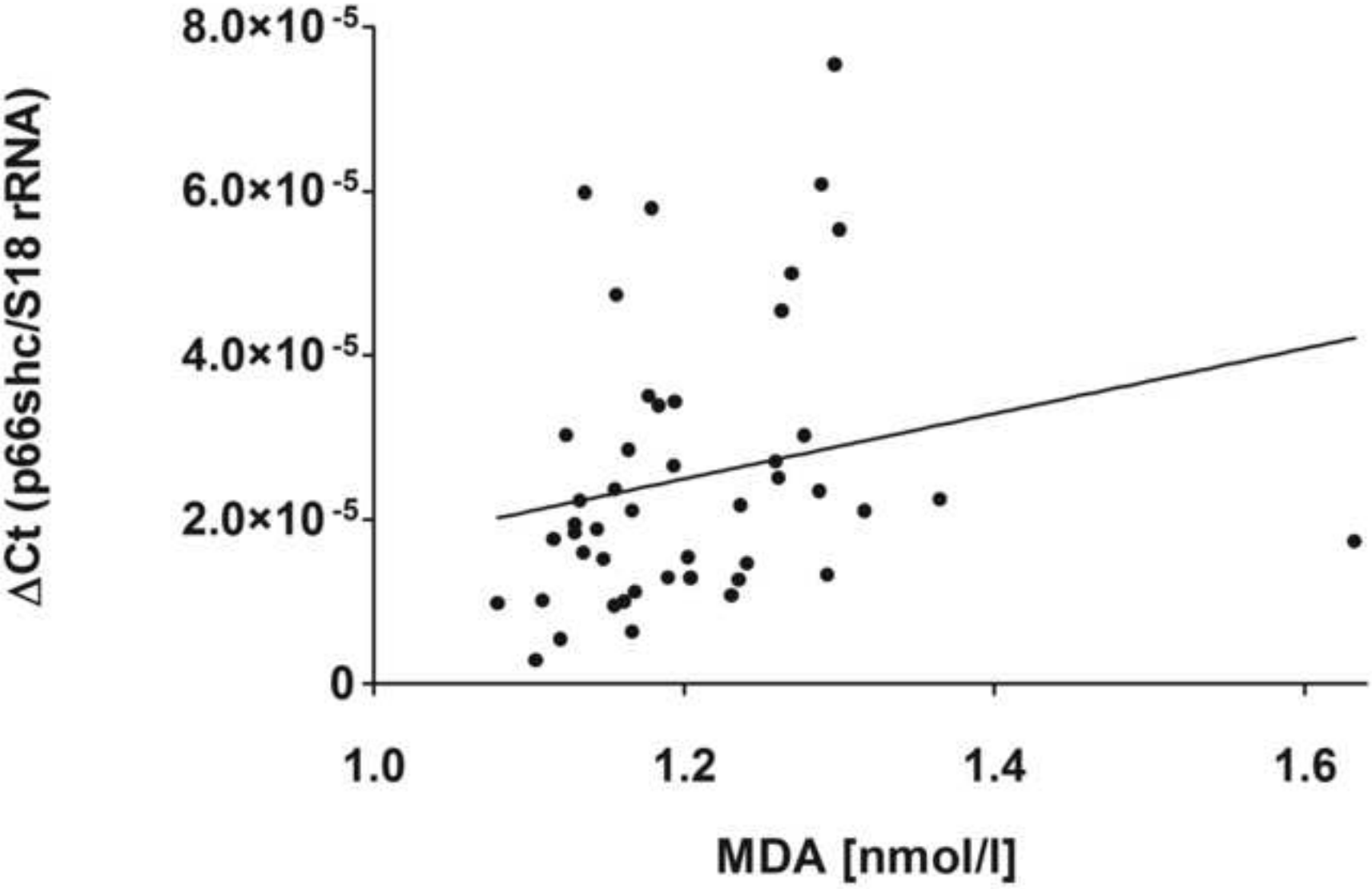

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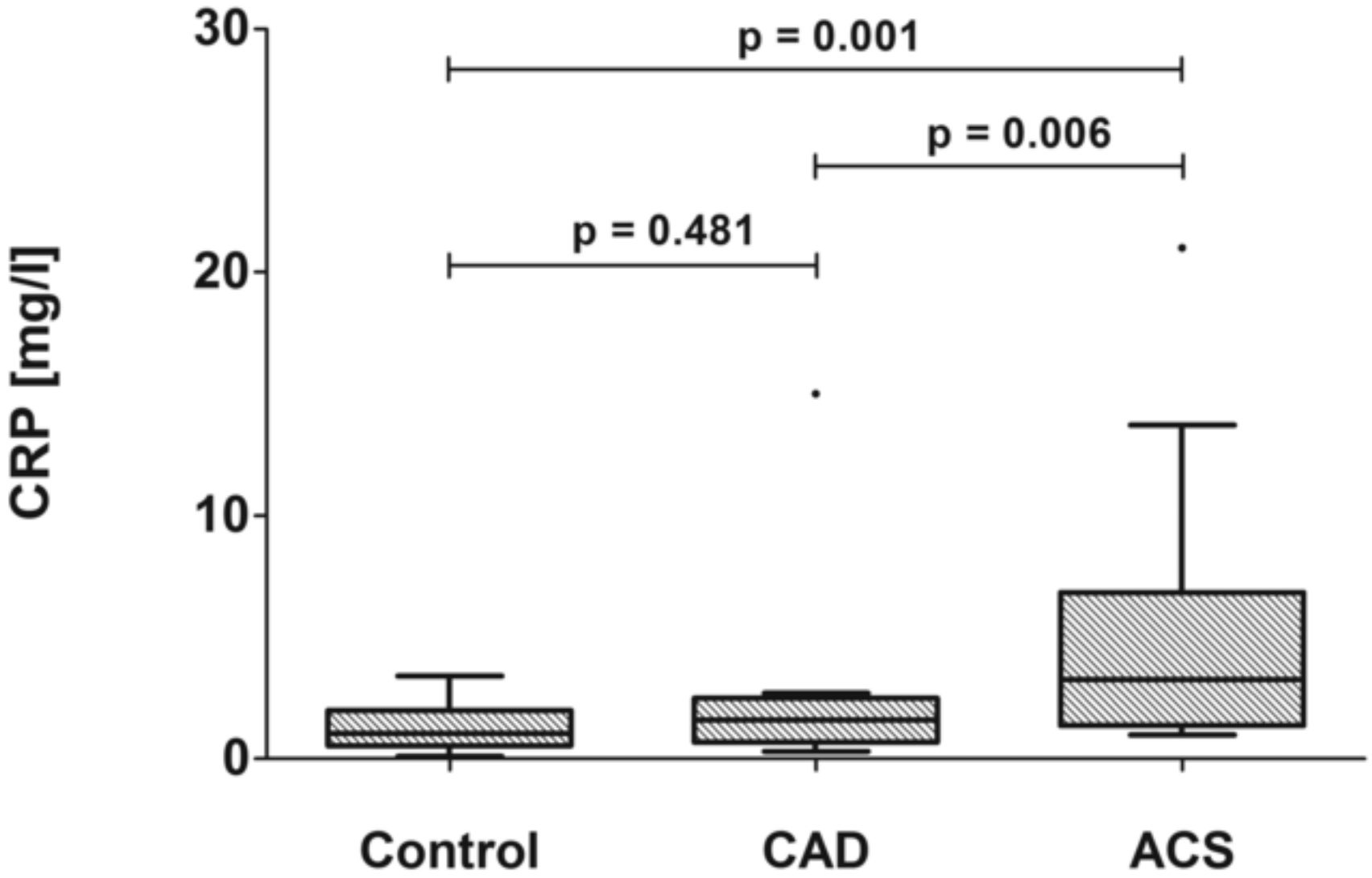

Table(s)

	Group		
	Control (n=16)	CAD (n=19)	ACS (n=18)
Gender (m/f)	16/0	19/0	18/0
Age (y)	60 (55-63)	61 (57-64)	59.5 (57-61)
BP syst. (mmHg)	134 (128-136)	135 (125-143)	132.5 (119-148)
BP diast. (mmHg)	80 (75-86)	78 (70-83)	83.5 (72-90)
BMI (kg/m²)	27.6 (24.8-29.8)	27.8 (24.5-31.1)	27.2 (25.1-30.4)
Glucose (mmol/l)	5.6 (5.4-6.1) *	5.9 (5.4-6.0) *	6.2 (5.9-6.8)
HbA1c	0.056 (0.055-0.059)	0.058 (0.056-0.060)	0.06 (0.057-0.061)
Total cholesterol (mmol/l)	4.45 (3.5-5.05)	4.2 (3.9-5.0) *	5.0 (4.5-6.0)
LDL cholesterol (mmol/l)	2.45 (1.98-3.07) *	2.3 (2.0-3.1) *	3.2 (2.8-4.1)
HDL cholesterol (mmol/l)	1.19 (1.08-1.34)	1.22 (0.94-1.47)	1.13 (0.9-1.37)
Triglycerides (mmol/l)	0.85 (0.66-1.44)	1.12 (0.75-1.86)	1.14 (0.84-1.79)
ALT (U/l)	28 (18-36)	31 (20-44)	38 (30-49)
CRP (mg/l)	1.1 (0.6-1.9) *	1.6 (0.7-2.5) *	3.3 (1.4-5.3)
Creatinine (µmol/l)	83 (78-94)	95 (82-98)	82.5 (78-108)
Hb (gr/dl)	14.4 (14.1-15.1)	14.4 (13.7-15.1)	14.4 (13.7-15.2)
Malondialdehyde (nmol/l)	1.186 (1.164-1.236) ● *	1.135 (1.12-1.161) *	1.261 (1.193-1.297)
p66shc (x10e-5,arbitrary units)	2.0 (1.19-2.78) *	1.73 (0.98-2.23) *	2.84 (2.1-5.53)
Hypertension	8 (50%)	13 (68%)	12 (67%)
Current smoking	4 (25%)	3 (16%)	10 (56%) ●
Dyslipidemia	5 (31%)	12 (63%)	11 (61%)
Obesity (BMI ≥ 30)	4 (25%)	6 (32%)	6 (33%)
Aspirin	8 (50%) ●	17 (89%)	10 (56%) ●
Clopidogrel	0 (0%) ●	7 (37%)	2 (11%)
ACE-inhibitor	9 (56%)	10 (53%)	9 (50%)
Betablocker	4 (25%)	9 (47%)	4 (22%)
Diuretics	3 (19%)	4 (21%)	2 (11%)
Statin	6 (38%) ●	16 (84%)	7 (39%) ●
Calcium antagonist	1 (6%)	3 (16%)	3 (17%)

Abbreviations: ACE-inhibitor, Angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; BMI, body mass index; BP diast., diastolic blood pressure; BP syst., systolic blood pressure; CRP, C-reactive protein; f, female; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; m, male; y, years.

Data are expressed as median (interquartile range) or count (percentage)

● $p < 0.05$ compared to CAD group

* $p < 0.05$ compared to ACS group